

Susceptibility of Human T Cell Leukemia Virus Type I to Nucleoside Reverse Transcriptase Inhibitors

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A single-cycle infection assay with recombinant viral vectors was developed to study human T cell leukemia virus type I (HTLV-I) replication and its inhibition by antiviral agents. The susceptibility of HTLV-I to 6 nucleoside reverse-transcriptase inhibitors was examined. HTLV-I replication was inhibited by tenofovir, abacavir, lamivudine, zalcitabine, stavudine, and zidovudine.

Human T cell leukemia virus type I (HTLV-I) is a retrovirus that infects ~20 million people worldwide, ~3% of whom develop adult T cell leukemia; a similar fraction develop a neurological disease known as HTLV-I-associated myelopathy—tropical spastic paraparesis (HAM-TSP) [1, 2]. Although there are currently no generally accepted therapies for these diseases, antiviral nucleoside analogs alone or in combination with interferon (IFN)- α have been tested in the clinic against HAM-TSP or adult T cell leukemia, respectively [3–6]. Antiviral agents specifically targeted against HTLV-I replication have not been developed, and, except for zidovudine [7–10], the susceptibility of HTLV-I to nucleoside reverse-transcriptase inhibitors (NRTIs) has not been tested in cell culture systems, mainly because of difficulties in establishing appropriate experimental methods for quantifying viral replication.

These problems were solved by the development of recombinant HTLV-I vectors that reproduce early steps of the viral

infectious cycle [7]. This sensitive and quantitative single-cycle replication system was used to examine the inhibition of HTLV-I replication by 6 antiviral nucleoside analogs. We were particularly interested in determining whether lamivudine could inhibit HTLV-I replication, because it is in clinical trials for treatment of HAM-TSP [6] and because its triphosphate form was reported to be inactive against HTLV-I reverse transcriptase (RT) in vitro [11].

Materials and methods. The plasmid clone encoding the infectious HTLV-I provirus, pHTLV-X1MT, has been described elsewhere [7]. Recombinant HTLV-I and human immunodeficiency virus (HIV) type 1 vectors for single-cycle replication assays have been described [7]. In the present report, the transfer vector, pHTC-GFP_{Luc}, encodes a green fluorescent protein (GFP)–luciferase fusion protein from the plasmid pEGFP_{Luc} (Clontech Laboratories). The HTLV-I packaging plasmid pCMVHT-YVDD was constructed by site-directed mutagenesis of pCMV-HT1 to encode a methionine to valine change in the YMDD motif of RT.

Human 293T cells were plated at 4×10^6 cells/10-cm dish 1 day before transfection by calcium phosphate coprecipitation. Wild-type HTLV-I virions were produced by transfecting cells with 10 μ g of the provirus clone, pHTLV-X1MT. Recombinant viruslike particles were generated by transfecting cells with 3 μ g of packaging plasmid (pCMVHT- Δ env), 3 μ g of transfer vector (pHTC-GFP_{Luc}), and 1 μ g of envelope expression plasmid (pCMV-VSVG). Cell culture supernatants were collected 48 h after transfection, filtered through 0.45- μ m low-protein binding filters (Millipore), and stored at -70°C before use. Virus concentrations in the filtered supernatants were determined by HTLV-I p19 ELISA (Zeptometrix) and were 50–75 ng/mL.

Human 293T cells and rhesus lung fibroblast (FRhL-clone B5) cells were maintained in Dulbecco modified Eagle medium plus 10% fetal calf serum. Single-cycle infections and luciferase assays were performed as described elsewhere [7]. The spreading infection assays [12] were initiated by infecting FRhL-B5 cells with 1.0 mL of supernatant from 293T cells transfected with pHTLV-X1MT. FRhL-B5 cells were seeded in 12-well plates at 1.2×10^5 cells/well the day before infection. Four days after infection, cells were transferred to 6-well plates at a dilution of 1:2; thereafter, cells were passaged at 3–4-day intervals in 6-well plates at a dilution of 1:5. Supernatants were collected at each passage for HTLV-I p19 ELISA. In drug-treated cultures, inhibitors were added 15 h before infection and were maintained throughout the experiment.

The following nucleoside analogs were added to target cells

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15 h before infection and were present throughout the remainder of the experiment: zidovudine (Zdv; 3'-azido-3'-deoxythymidine; Sigma Chemical), lamivudine (3TC; 2',3'-dideoxy-3'-thiacytidine; Moravek Biochemicals), stavudine (d4T; 2',3'-didehydro-3'-deoxythymidine; Sigma), zalcitabine (ddC; 2',3'-dideoxycytidine; Sigma), abacavir (ABC; 1592U89; Moravek), and tenofovir (Tfv; bis-POC-PMPA; Moravek). Luciferase activities in cell extracts were determined 72 h after infection. To ensure that the agents did not exert toxic effects on the cells and to normalize luciferase activities, protein concentrations of cell extracts were determined by the bovine serum albumin protein assay method (Pierce). Four concentrations of each compound, as well as a no-drug control, were tested in duplicate. IC_{50} values were calculated by plotting the percentage luciferase activity, relative to no-drug control, versus \log_{10} drug concentration. Experimental points were plotted by linear regression analysis, and IC_{50} values were calculated from the plots. Mean IC_{50} values for each nucleoside analog were calculated from at least 3 independent experiments.

Results. A single-cycle replication assay was developed for HTLV-I to monitor virus entry, reverse transcription, and genome integration steps of the virus infectious cycle [7]. Recombinant virus stocks were produced by cotransfecting human 293T cells with an HTLV-I packaging plasmid (pCMV-HT1Δenv), a transfer vector (pHTC-GFP-Luc) and an envelope expression plasmid (pCMV-VSVG). The packaging plasmid expresses viral structural, enzymatic, and regulatory proteins, but its mRNA cannot be packaged into virus particles; it also lacks an *env* gene, so that the particle can be pseudotyped. An envelope expression plasmid provides the glycoprotein envelope required for virion binding and entry into the target cell. The transfer vector expresses a surrogate HTLV-I genomic mRNA containing a cytomegalovirus promoter and GFP-luciferase fusion protein cassette, which is packaged into virions and is replicated in the infected cell. Expression of luciferase activity in infected cells is proportional to the amount of recombinant virus used for infection and is dependent on the successful completion of entry, reverse transcription, and integration processes [7].

The NRTIs 3TC, ABC, Tfv, d4T, Zdv, and ddC were tested in the single-cycle HTLV-I replication assay. Luciferase activity, expressed relative to no-drug control, was plotted versus \log_{10} NRTI concentration to derive IC_{50} values (figure 1). HTLV-I was susceptible to all 6 NRTIs tested. Tfv was the most potent inhibitor of HTLV-I replication, with an IC_{50} of 5.4 nmol/L, followed by Zdv (IC_{50} , 0.11 μ mol/L) and ddC (IC_{50} , 0.27 μ mol/L). The IC_{50} value for ABC was 4.6 μ mol/L. d4T and 3TC were the least potent of the drugs tested against HTLV-I, with IC_{50} values of 14.5 μ mol/L and 22.0 μ mol/L respectively. These compounds displayed a similar pattern of activity against HTLV-I replication when tested in human osteogenic sarcoma (HOS) cells and human C8166 T cells (data not shown); IC_{50} values

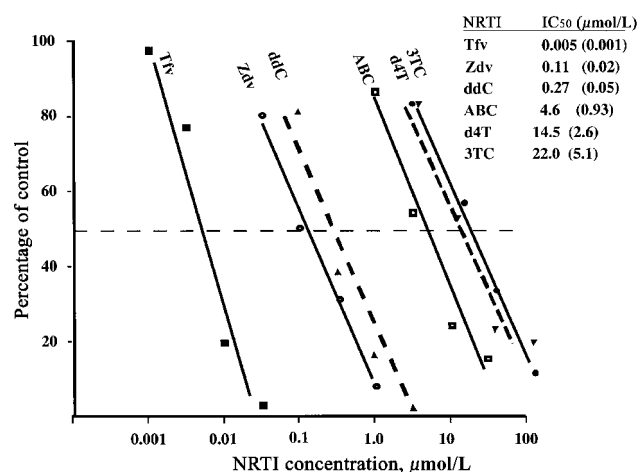


Figure 1. Inhibition of human T cell leukemia virus type I (HTLV-I) replication by nucleoside reverse-transcriptase inhibitors (NRTIs) in single-cycle infection assays. Filtered supernatants from transiently transfected 293T cells (recombinant virus producers) were used to infect FRhL-B5 cells (virus targets). Recombinant virus infection and replication in the target cells was determined by performing luciferase assays on cell extracts 72 h after infection. Cells were treated with varied concentrations of nucleoside analogs for 15 h before infection and maintained with drugs during and after infection. Luciferase activities are expressed as the percentage of activity relative to no-drug controls and are plotted against the \log_{10} of the drug concentration (μ mol/L). Best-line fitting was performed by linear regression analysis. *Inset*, IC_{50} values were determined from each plot; the mean values with SDs in parentheses are shown. 3TC, lamivudine; ABC, abacavir; d4T, stavudine; ddC, zalcitabine; Tfv, tenofovir; Zdv, zidovudine.

varied among cell lines as a result of their differing abilities to metabolize the NRTIs to their active triphosphate forms [13].

The inhibition of HTLV-I replication by 3TC in the single-cycle replication assay was confirmed in a spreading infection system with wild-type virus. FRhL-B5 cells were infected and maintained in the presence of various concentrations of 3TC (figure 2A). Virus expression was monitored by p19 (*gag*) ELISA of cell culture supernatants at 3- or 4-day intervals after infection. 3TC-inhibited virus spread through multiple rounds of replication. At 18 days after infection, virus expression was inhibited 76% by 5 μ mol/L 3TC, 88% by 10 μ mol/L 3TC, and 99% by 20 μ mol/L 3TC.

A methionine to valine mutation in the highly conserved YMDD motif of HIV-1 RT confers resistance to 3TC [14]. To establish that the inhibitory effect of 3TC on HTLV-I replication was mediated by RT, we constructed an HTLV-I mutant containing the analogous methionine to valine mutation in the YMDD motif of the enzyme's active site. When tested in the single-cycle infection assay, the HTLV-I YVDD mutant was resistant to 3TC (figure 2B). In the absence of 3TC, the wild-type and YVDD mutant viruses gave nearly identical levels of infection and showed similar sensitivities to Zdv.

Discussion. HTLV-I replication was inhibited by the 6

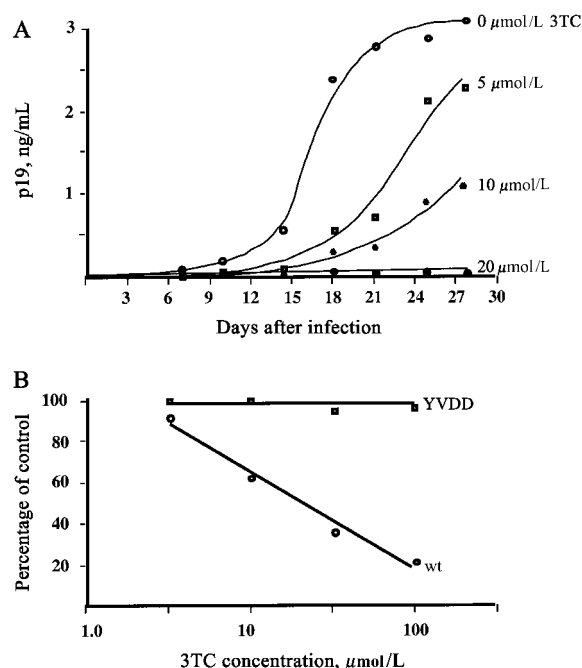


Figure 2. Human T cell leukemia virus type I (HTLV-I) reverse transcriptase is the target of 3TC inhibition. *A*, Replication of wild-type (wt) HTLV-I was monitored in spreading infection assays of FRhL-B5 cells maintained in the indicated concentrations of lamivudine (3TC). Aliquots of culture medium were analyzed by HTLV-I p19 (*gag*) ELISA at biweekly intervals. *B*, A mutated HTLV-I packaging plasmid (designated as YVDD) was constructed that encodes a reverse transcriptase (RT) with a methionine to valine change in the active site of the enzyme. The analogous mutation in human immunodeficiency virus type 1 RT confers resistance to 3TC [14]. Viruslike particles generated from wt and YVDD mutant packaging plasmids were tested in single-cycle replication assays of FRhL-B5 cells. In the absence of 3TC, the recombinant viruses gave nearly identical levels of transduction. Infections were performed in the presence of the indicated concentrations of 3TC and are plotted as the percentage activity remaining relative to no-drug controls.

NRTIs that were examined here. Tfv was the most potent inhibitor, whereas d4T and 3TC required much higher concentrations of drug to achieve 50% inhibition. Of these nucleoside analogs, only Zdv has been tested previously as an inhibitor of HTLV-I replication in cell culture systems [7–10]. Although earlier studies of Zdv inhibition of HTLV-I replication in primary human lymphocytes did not establish IC_{50} values [8, 10], the dose-response observed with wild-type virus is consistent with IC_{50} values determined here. HTLV-I was inhibited by 3TC both in single-cycle and spreading infection assays. That HTLV-I RT was the target of this inhibitory effect was demonstrated by the 3TC-resistant phenotype of the HTLV-I mutant, which had a methionine to valine change in the active site of RT.

The susceptibility of HTLV-I to 3TC in our infectivity assays stands in contrast to a report suggesting that HTLV-I RT was resistant to the triphosphate form of 3TC (3TC-TP) in an *in vitro* enzyme assay [11]. It is likely that the lack of inhibition

of HTLV-I RT by 3TC-TP *in vitro* was due to the reaction conditions (10 $\mu\text{mol/L}$ 3TC-TP and 5 $\mu\text{mol/L}$ dCTP) combined with a relatively low affinity of 3TC-TP for HTLV-I RT. Under the same *in vitro* reaction conditions, ddC-TP gave an IC_{50} of ~ 1 $\mu\text{mol/L}$. Because the IC_{50} for 3TC in cell culture assays was ~ 100 -fold higher than ddC, it is not surprising that the IC_{50} for 3TC-TP *in vitro* would be >10 $\mu\text{mol/L}$, as reported elsewhere [11]. In its susceptibility to 3TC, HTLV-I more closely resembles HIV-1 than murine leukemia virus, which is resistant to 3TC [15]. The NRTI IC_{50} values determined here for HTLV-I were similar to IC_{50} values determined in parallel (authors' unpublished observations) or reported for HIV-1 [15, 16].

There have been several reports on the use of Zdv [3–5, 17] and one on the use of 3TC [6] to treat adult T cell leukemia or HAM-TSP. In a study of 19 patients with advanced adult T cell leukemia-lymphoma, treatment with a combination of IFN- α plus Zdv was reported to elicit major responses in 58% and complete remission in 26% of the individuals [3]. In a study of patients with HAM-TSP, provirus loads were monitored during the course of treatment and were shown to decrease abruptly after initiation of 3TC therapy [6]. In the latter study, provirus load rebounded during therapy, which may underscore a potential problem in the use of NRTIs alone against HTLV-I-associated diseases. HTLV-I provirus load probably reflects both infectious spread of the virus, as well as clonal expansion of virus-infected cells. Because NRTIs inhibit only the infectious spread of the virus, they would have to be administered in combination with antineoplastic agents or immune modulators to achieve a sustained decrease in the total virus load.

The single-cycle replication assay described here is widely applicable to screening and mechanistic studies of anti-HTLV-I drugs and should facilitate the development of specific, highly active inhibitors directed against this virus. In addition to reverse-transcriptase inhibitors, this system can easily be extended to the study of other classes of antiviral compounds directed against virus enzymes such as integrase or protease. The vectors described here also provide a method for phenotypic analyses of drug-resistant mutants that could potentially arise during antiviral therapy.

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Addendum. Balestrieri et al. [18] recently reported that lamivudine inhibited human T cell leukemia virus type I replication in primary human lymphocytes in vitro at concentrations consistent with the IC₅₀ values reported here.